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RNA Interference with Measles Virus N, P, and L mRNAs Efficiently Prevents and with Matrix Protein mRNA Enhances Viral Transcription

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In contrast to studies with genetically modified viruses, RNA interference allows the analysis of virus infections with identical viruses and posttranscriptional ablation of individual gene functions. Using RNase III-generated multiple short interfering RNAs (siRNAs) against the six measles virus genes, we found efficient downregulation of viral gene expression in general with siRNAs against the nucleocapsid (N), phosphoprotein (P), and polymerase (L) mRNAs, the translation products of which form the ribonucleoprotein (RNP) complex. Silencing of the RNP mRNAs was highly efficient in reducing viral messenger and genomic RNAs. siRNAs against the mRNAs for the hemagglutinin (H) and fusion (F) proteins reduced the extent of cell-cell fusion. Interestingly, siRNA-mediated knockdown of the matrix (M) protein not only enhanced cell-cell fusion but also increased the levels of both mRNAs and genomic RNA by a factor of 2 to 2.5 so that the genome-to-mRNA ratio was constant. These findings indicate that M acts as a negative regulator of viral polymerase activity, affecting mRNA transcription and genome replication to the same extent.

Measles virus (MV), a member of the family *Paramyxoviridae* and the genus *Morbillivirus*, has six genes, resulting in eight proteins. Relative frequencies of the viral mRNAs follow the transcriptional gradient in the genomic order N-P-M-F-H-L, with approximately 5- to 250-fold more N mRNA than L mRNA, depending on the host cell type (14, 53). We wanted to inhibit single viral gene functions by using a method that allows the comparison of the effects on the viral gene expression and the replication of the genome on an identical viral and cellular background. This can be achieved exactly by the recently established short interfering RNA (siRNA) technology, which does not require the generation of recombinant viruses bearing mutations or lacking a gene, which usually results in viruses with different growth properties. Not all viral protein functions of nonsegmented negative-strand RNA viruses are unambiguously defined. Various findings have been published concerning functions of the matrix (M) proteins of vesicular stomatitis virus (VSV), rabies virus, respiratory syncytial virus (RSV), and other viruses, which not only play a role in virus budding but may also act as transcriptional inhibitors of the viral polymerase and could affect mRNA transcription and/or genome replication or the balance between transcription and replication (1, 2, 23, 24, 27, 45, 50). In the case of MV, the addition of the matrix protein to in vitro transcription assays with isolated ribonucleoproteins (RNPs) from which the M protein was biochemically removed provided the first hints that M acts as an inhibitor of viral transcription (54). A matrix-deficient recombinant MV was found to be more efficient in inducing cell-to-cell fusion, but virus titers were reduced (13). Interestingly, this virus lost acute pathogenicity but spreads more efficiently in the brain of CD46-transgenic interferon (IFN) receptor-deficient mice, probably due to its enhanced fusogenicity (13). Using siRNA, we analyzed the M protein-dependent regulation of viral mRNA and genomic transcription in cells infected with identical viruses and with minimal alterations within the host cell.

RNA interference (RNAi) plays a role in antiviral defense in plants (11), and, vice versa, viruses have evolved mechanisms to suppress it (39). Experimentally, it is an excellent means to knock down and analyze the function of individual genes (16, 19, 29, 41, 57). RNAi has been used successfully to inhibit a number of viral infections, including human immunodeficiency virus type 1, influenza A virus, West Nile virus, hepatitis B and C viruses, and RSV (6, 7, 21, 26, 36, 38, 44, 56; for a review, see reference 10). There are several ways in which functional siRNAs can be generated and introduced into target cells. Interfering RNAs can be synthesized as short, single-stranded RNAs or produced as short hairpin RNAs transcribed from plasmids or viral expression systems or by cutting longer (300- to 800-bp) double-stranded RNA (dsRNA) fragments into pools of multiple ≈22-bp siRNAs by RNase III (Dicer) (28, 32).

Since we wanted to compare the effects of siRNAs against the six MV gene-specific mRNAs, we did not use single siRNAs, which vary considerably in their efficiency in a sequence-dependent way, but we used evenly effective RNase III-produced multiple siRNAs. Because of the transcriptional gradient of negative-strand RNA viruses, one may hypothesize that siRNAs against the least frequent mRNA, the polymerase mRNA, may be the most effective, whereas siRNAs against the most frequent mRNAs, N and P, may be less effective. However, we found N-, and L-specific siRNAs to inhibit viral gene expression equally well. Using M-specific siRNA, we demonstrate that the reduction of M mRNA and protein levels leads to increased levels of other viral mRNAs, corresponding proteins, and genomic RNA. This suggests that M affects polymerase activity in general, with an equal effect on mRNA transcription and genome replication.

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MATERIALS AND METHODS

Cells, viruses, and antibodies. CHO-CD46 (CHO-5.3; a gift of B. Loveland, Australia) and CHO-SLAM (22) Chinese hamster ovary cells and African green monkey kidney Vero cells were cultivated in modified Eagle's medium containing 5% fetal calf serum. The attenuated MV strain Edmonston B (Edm) was propagated using Vero cells. Wild-type strain WTFb (Erlangen.DEU/90) was propagated using the human B-cell line BJAB as described previously (22). Supernatants containing monoclonal antibodies (mAbs) against the MV N protein (clone F227), MV P protein (clone P3), MV F protein (clone A504), and MV H protein (clone K83) were harvested from hybridomas and purified using protein G columns. The monoclonal antibodies against the MV M protein (MAB8910) were purchased from Chemicon. Polyclonal sera raised in rabbits against the cytoplasmic sequences of the F and H proteins (H_2N-CPDLTGTSK SYVRSL-$CONH_2$ and H_2N-SPQRDRINAFYKDNC-$CONH_2$) coupled to keyhole limpet hemocyanin were obtained from Eurogentech. The β-tubulin mAb was purchased from Abcam. As a secondary antibody in the immunofluorescence assay, we used fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (Dianova) or rabbit anti-mouse immunoglobulins conjugated to FITC (DAKO). As secondary antibodies for Western blotting, we used horseradish peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulin (Jackson Immunoresearch Laboratories). Mx proteins are exclusively induced by IFN-α/β. Their detection was used as a highly sensitive indicator of the induction of endogenous IFN-α/β (52). We used mAb 143 for the detection of the 78-kDa human MxA and rodent Mx proteins on Western blots, which has been raised in our laboratory and the binding site of which has been mapped on MxA(25).

Synthesis and transfection of siRNA. Multiple siRNAs specific for the six MV genes were generated from several-hundred-base-pair-long double-stranded RNAs synthesized by T7 polymerase. Plasmids pSC-N, pSC-P, pSC-M, pCG-F1, pCG-H5, and pEMC-La (30, 33, 48), bearing the sequences coding for the viral proteins, were used as PCR templates. We used primers containing the T7 polymerase promoter sequence fused with viral gene-specific sequences (5′-TA ATACGACTCACTATAGG-specific primer-3′) to generate PCR fragments with T7 promoters at both ends. The viral gene-specific parts of the primers and locations in the MV Edm genome are as follows: for pSC-N, 5′-ACCCATTAC ATCAGGATCCGGT-3′ (forward) and 5′-TGAAAAGTATTGGTCCGCCTC AT-3′ (reverse) (positions 161 to 504); for pSC-P, 5′-ACCCAATGTGCTCGA AAGTC-3′ (forward) and 5′-GGCCTGAATCTCTGCCTATG-3′ (reverse) (positions 2560 to 3016); for pSC-M, 5′-AGAGTCATAGATCCTGGTCTAGG CG-3′ (forward) and 5′-CTCAGGAAGTTGCTCTGTATTGTCG-3′ (reverse) (positions 3543 to 4079); for pCG-F1, 5′-GTGCAAGCTACAAAGTTATGAC TCGTT-3′ (forward) and 5′-TCACAAGATAGTTGGTTCATAGACGGT-3′ (reverse) (positions 5576 to 6044); for pCG-H5, 5′-TGTCTCTGAGCTTGATC GGGTT-3′ (forward) and 5′-AAGTCTAACAGGGACAGCGACATG-3′ (reverse) (positions 7404 to 7893); and for pEMC-La, 5′-CCTCAAAAAGGGGA ATTCGCT-3′ (forward [L1]) and 5′-TTCATCAGAAAACCCGTTTTGG-3′ (reverse [L1]) (positions 9560 to 10205) and 5′-GCAAGGTTTGTCCTGATCC A-3′ (forward [L2]) and 5′-GGACCTCAAGGCCGTAAATA-3′ (reverse [L2]) (positions 12283 to 12711). T7 RNA polymerase (MBI Fermentas) was used to transcribe RNAs in both orientations from these DNA templates according to the manufacturer's instructions. Products were analyzed for their correct length by 1% agarose gel electrophoresis. The large dsRNA fragments were digested with RNase III (Shortcut; New England Biolabs). Digested siRNAs were then column purified using the RNeasy kit from QIAGEN (9). Purified siRNAs were quantified by UV absorbance and analyzed by gel electrophoresis using 3% high-resolution agarose (QA-agarose; Qbiogene). Unless otherwise indicated, cells (4.5×10^4) in 24-well plates were transfected with 400 ng siRNA in 2 μl/well using the HiPerFect transfection reagent (QIAGEN).

Immunofluorescence analysis. Cells were grown on eight-chamber slides (Nunc), transfected with siRNA, and infected as indicated. Cells were fixed 48 h later with 4% paraformaldehyde and permeabilized with 0.5% Triton X-100. Immunostaining was performed according to standard protocols. As a secondary antibody, we used FITC-conjugated rabbit anti-mouse immunoglobulins. Nuclei were stained using DAPI (4′,6′-diamidino-2-phenylindole dihydrochloride; Molecular Probes). Photomicrographs were taken with a Leica DM IRE2 fluorescence microscope and digital camera.

Polyacrylamide gel electrophoresis and Western blotting. Cells were lysed in loading buffer (50 mM Tris HCl [pH 6.8], 2% sodium dodecyl sulfate [SDS], 10% glycerol, 1% β-mercaptoethanol, 12.5 mM EDTA, 0.02% bromophenol blue) and analyzed by standard Western blotting procedure. Briefly, samples were separated by 10% SDS-polyacrylamide gel electrophoresis and blotted semidry on nitrocellulose (Schleicher & Schuell). Filters were blocked with 5% dry milk (Fluka) in phosphate-buffered saline containing 0.05% Tween 20 and incubated with MV-specific antibodies and horseradish peroxidase-conjugated secondary antibodies. Proteins were visualized using the ECL system (Amersham) and quantified using an LAS-3000 luminescent image analyzer (Fuji film).

Quantification of MV genomes and mRNAs by real-time RT-PCR. Total RNA was extracted 48 h postinfection from 2.5×10^5 cells using the RNeasy kit (QIAGEN). First-strand cDNA synthesis from 200 ng RNA was primed with random hexamers for the MV genome, or oligo(dT) for viral mRNAs, and polymerized with Moloney murine leukemia virus reverse transcriptase (RT; Promega). The measles-specific primers were MV-N forward (5′-CCCTCTGC TCTGGAGCTATG-3′) (position in the MV Edm genome, nucleotide [nt] 1094), MV-N reverse (5′-GCCAATGTGGAACTGACCTT-3′) (nt 1249), MV-P forward (5′-ATCAGACAACCCAGGACAGG-3′) (nt 1923), MV-P reverse (5′-AGTGCTTGATGCCTGGAGAT-3′) (nt 2121), MV-M forward (5′-GAGCAA CTTCCTGAGGCAAC-3′) (nt 4065), MV-M reverse (5′-TTGTGCATGGAG AGTCTTGC-3′) (nt 4256), genomic RNA (as described previously [47]), MV-L-trailer forward (5′-GAGAAACAGATTATTATGACGGG-3′) (nt 15668), MV-L-trailer reverse (5′-CAAAGCTGGGAATAGAAACT-3′) (nt 15888), and hamster-specific GAPDH (CHO cells) forward (5′-GTATTGGACGAATGGTTA CCA-3′) and reverse (5′-GGTAGAGTCATACTGGAACATGTAGACC-3′) (PCR product, 976 bp). A standard curve was generated by pooling the undiluted cDNAs and a series of 10-fold dilutions. Reactions were carried out in duplicate in 96-well thin-wall PCR plates (Bio-Rad) using the iCycler iQ Multicolour real-time detection system (Bio-Rad). The PCR protocol consisted of an initial 10-min polymerase activation step at 95°C followed by a 30-s denaturation step at 95°C, a 30-s annealing step at 60°C, and a 30-s extension step at 72°C, repeated for 40 cycles. Following the final extension step, the PCR products were denaturated once more at 95°C for 1 min and reannealed at 55°C for 1 min, and a melting curve was generated. Analysis of the melting curve indicated the presence of only a single product in each reaction, which was confirmed after the products were resolved on a 1.5% agarose gel. The efficiency (E) of each PCR primer pair was determined from the slope of the standard, according to the equation $E = 10^{(-1/\text{slope})}$. Efficiencies were 90 to 98%. Quantification of the transcripts was carried out according to the mathematical model described previously by Pfaffl (46). Transcript levels are normalized to the reference glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene and are expressed as a relative expression ratio (R), according to the equation $R = E_x\Delta C_{T_x}/E_{\text{ref}}\Delta C_{T_{\text{ref}}}$, where x is the gene of interest, ref is the reference gene, GAPDH, ΔC_T is the difference in the cycle thresholds (C_T) between the control and treatment samples, and E is the real-time PCR efficiency for the given primer pair (46).

RESULTS

Selection of sequences and synthesis of siRNA. In order to analyze the functional consequences of gene-specific siRNAs, we selected a region within each viral gene to generate gene-specific multiple siRNAs by RNase III digestion (Fig. 1A). Multiple siRNAs were produced from longer double-stranded RNAs transcribed by T7 RNA polymerase as described in Materials and Methods. The amount and the quality of all preparations of siRNAs were controlled by agarose gel electrophoresis. Examples of T7 polymerase-generated long dsRNA molecules corresponding to parts of the N, P, M, F, H, and L genes are shown in Fig. 1B, and purified siRNAs generated from these long dsRNAs are shown in the same order in Fig. 1C.

We controlled the lack of interferon type I (IFN-α/β) induction after siRNA transfections by using a highly specific test based on the induction of Mx proteins as a surrogate marker for the presence of IFN-α/β. Since CHO-CD46 cells do not respond to human IFN-α/β (hIFN-α/β), we used the above-described long dsRNA as a positive control for the induction of endogenous IFN-α/β and Mx. Forty-eight hours after transfection with undigested dsRNA of 456 bp, the cells expressed the 78-kDa Mx protein (Fig. 1D, lane 2). In contrast, similar amounts of the purified siRNA samples did not induce the expression of Mx (Fig. 1D, lanes 3 and 4). Several siRNA preparations were tested in this assay, and no induction of Mx

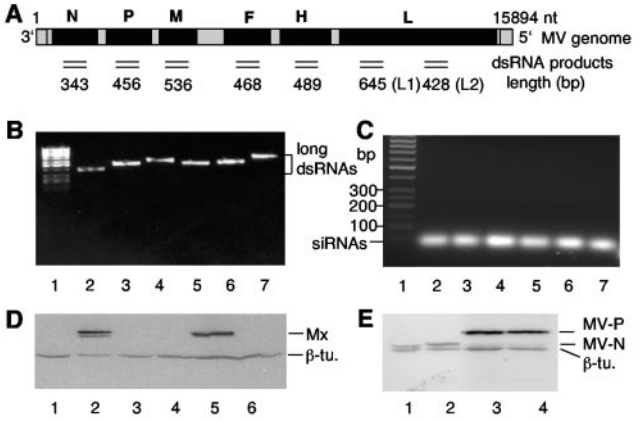

FIG. 1. Selection of sequences of the MV genome for shortcut siRNA production and quality of siRNA. (A) The lengths of the MV gene-specific PCR products, from which long dsRNAs were transcribed with T7 polymerase, are shown. (B and C) Long dsRNA molecules corresponding to N, P, M, F, H, and L1 (lanes 2 to 7) (B) and purified siRNAs in the same order (lanes 2 to 7) (C) on a 3% high-resolution agarose gel. (D) Lack of IFN-α/β induction by siRNA transfection. The expression of the IFN-α/β-inducible Mx protein was investigated in cells transfected with siRNA and with long dsRNA as a control. CHO-CD46 cells treated with human IFN-α (1,000 U/ml) do not respond due to species diversity (lane 1). CHO-CD46 cells transfected with 0.3 μg 456-bp-long dsRNA (lane 2) and CHO-CD46 cells transfected with 0.3 μg purified siRNA (lane 3) are shown. Human BJAB cells, known to express MxA in response to hIFN-α, were used as a positive control. BJAB cells transfected with 0.3 μg purified siRNA (lane 4), BJAB cells treated with 1,000 U hIFN-α for 24 h (lane 5), and untreated BJAB cells are shown (lane 6). (E) Absence of an effect on viral gene expression after transfection of cells with unrelated siRNA (CD150-siRNA). Cells were infected and not transfected (lanes 1 and 3) or transfected with unrelated siRNA (lanes 2 and 4). Protein extracts were blotted onto nitrocellulose, and the viral N (lanes 1 and 2) and P (lanes 3 and 4) proteins were detected with monoclonal antibodies. As a control for the amount of protein present on the nitrocellulose filter, β-tubulin (β-tu.) is shown in addition to the other proteins analyzed.

was observed. As a further control for the lack of unspecific effects, we used an unrelated siRNA against a cellular mRNA (CD150) not expressed in CHO-CD46 cells. The expression of the viral N and P proteins was assessed after transfection and infection of the cells with MV Edm. The unrelated siRNA did not influence the expression of viral proteins (Fig. 1E).

***cis* and *trans* effects of siRNAs on viral gene expression.** To demonstrate effects of the viral gene-specific multiple siRNAs, we first transfected cells with siRNA preparations against the N, P, and L viral RNP genes. An unrelated siRNA was used as a control. Cells were fixed and stained with antibodies to detect the viral proteins targeted by the corresponding siRNAs in the case of N and P (*cis* effect). As no L-specific antibody was available, the effect of L siRNA on viral gene expression in general was assessed using antibodies to the N protein (*trans* effect). We observed a significant reduction in the expression of N and P after treatment of the cells with N, P, and L siRNAs, respectively (Fig. 2). By analyzing the envelope proteins, we found similarly pronounced effects of the M, F, and H siRNAs on the expression of the corresponding proteins (data not shown). The effect on the morphology of cells was examined by phase-contrast microscopy (Fig. 3). In the case of M siRNA, the formation of syncytia was enhanced (Fig. 3B), whereas F and H siRNAs prevented the formation of syncytia, as expected (Fig. 3C and D). An almost complete inhibition of virus-induced cell fusion was also found with siRNAs against the RNP mRNAs (N, P, and L) (data not shown).

The *cis* effect of the multiple siRNAs against different viral mRNAs was further analyzed at the protein level by Western blotting. Cells were transfected with the siRNAs and infected with MV Edm 24 h later, and cell lysates were prepared 48 h later. Strong inhibitory effects of N, P, M, F, and H siRNAs on the expression of the corresponding proteins were detected (Fig. 4A) (effect on F was not shown). Codetection of β-tubulin served as a loading control.

In order to investigate the *trans* effects of the RNP-specific siRNAs on viral gene expression in general, we transfected cells with multiple siRNAs against N, P, and L and analyzed the expression of P, N, and P, respectively (Fig. 4B). This, and the results described above, revealed that the siRNAs against viral mRNAs for proteins constituting the RNP complex had a strong inhibitory effect on viral gene expression in general. When cells were treated with siRNA against M mRNA, expression of the other viral proteins was markedly enhanced (Fig. 4C). We demonstrate this for N, P, F, and H proteins by using Western blots (Fig. 4, arrowheads) in each case, with M and β-tubulin expression as the internal control. A quantification of the proteins detected on Western blots is shown in Fig. 4D. The intensities of the protein bands from untreated cells was set to 100%. The levels of M were reduced by M siRNA to various extents in the individual experiments, whereas levels of the other proteins increased between 2- and 3.5-fold. Similar effects were observed using CHO-SLAM cells and a wild-type MV strain (strain WTFb). Also, in wild-type MV-infected cells, M siRNA induced increased expression of other viral proteins (data not shown).

Effect of siRNAs on viral genomic and mRNA levels. In order to investigate whether a reduction of the M protein level affects the transcription of viral mRNAs and/or the genome replication simultaneously or selectively, we performed real-time RT-PCR analyses from total RNA preparations of cells transfected with various siRNAs. Results of three independent experiments are presented in Fig. 5 as relative expression levels of the RNAs in relation to the positive control (cells transfected with unrelated siRNA) and corrected for equal GAPDH mRNA levels. In the first experiment, the viral P and M mRNAs following treatment of the cells with N, M, L1, and L2 siRNAs were analyzed (Fig. 5A and B). In cells transfected with N siRNA, mRNAs for M and P were reduced by a factor of approximately 9. In the case of M siRNA, a fivefold inhibition of the M mRNA levels and an approximately 2.2-fold stimulation of the P mRNA level were observed. We then determined the effect of L-specific siRNAs (L1 and L2 siRNAs) on P and M mRNA levels (Fig. 5 A and B). Both siRNAs reduced the level of the mRNAs by five- to ninefold.

The levels of viral genomes were assessed in cells transfected with N, M, L1, and L2 siRNAs (Fig. 5C). In the presence of N, L1, and L2 siRNAs, we detected approximately fivefold less genomic RNA, whereas in the presence of M siRNA, there was approximately twofold more genomic RNA. Thus, N and L siRNAs efficiently inhibited viral mRNAs and genomic RNA levels, whereas silencing of M enhanced the expression of the other viral mRNAs and genomic RNA.

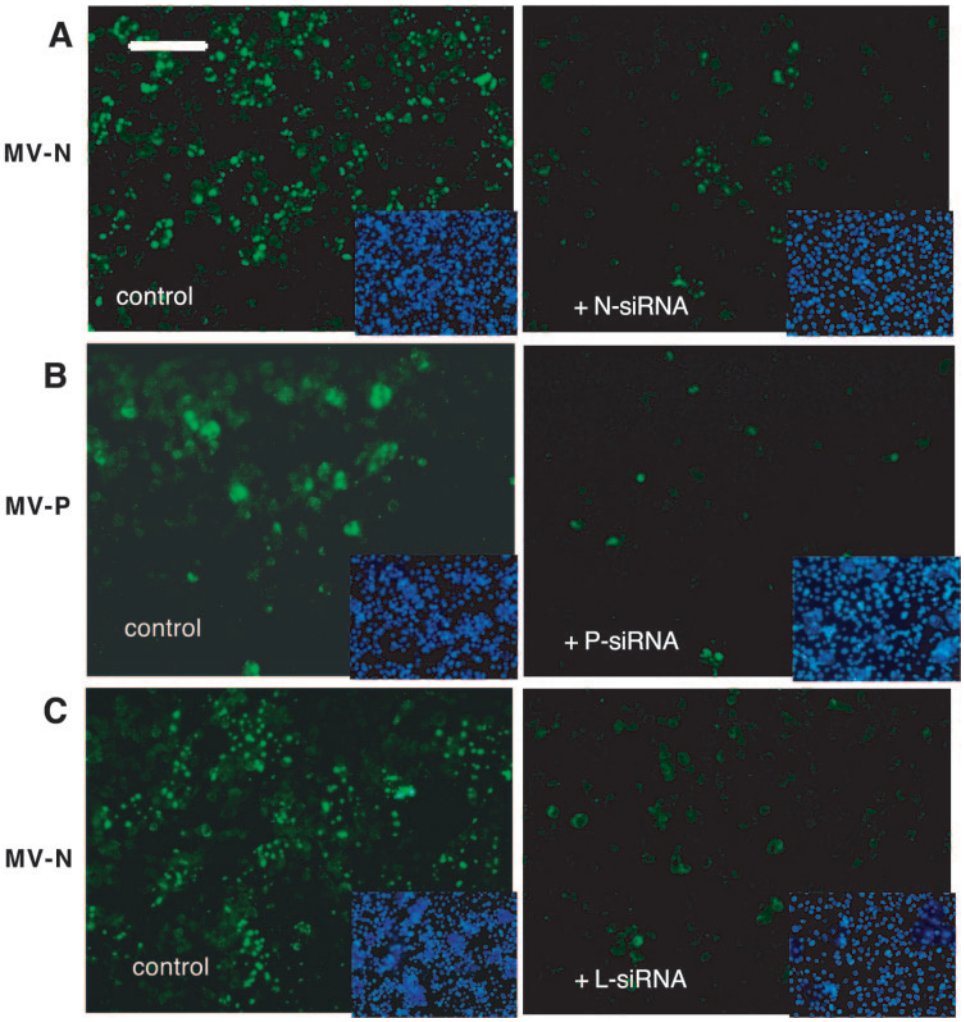

FIG. 2. Effect of RNP-specific siRNAs on the expression of N and P in MV-infected cells. CHO-CD46 cells were transfected with shortcut-produced unrelated control siRNA (left panels) or siRNAs against viral mRNAs (right panels, as indicated), infected with MV Edm at a multiplicity of infection of 0.5, and fixed for immunostaining 48 h later. Cells were stained with mAbs against MV N (A), MV P (B), and MV N (C) and secondary FITC-conjugated antibodies and counterstained with DAPI. (Magnification, ×20; bar in A, 20 μm.) Inserts show the corresponding DAPI-stained fields as a control for the amount of cells.

DISCUSSION

The application of multiple viral gene-specific siRNAs prior to infection of cells with MV inhibited the expression of corresponding viral mRNAs and proteins very effectively. MV gene expression and genomic RNA replication in general were inhibited with siRNAs against mRNAs encoding proteins of the RNP complex, N, P, and L. Interestingly, RNA interference with viral mRNAs was highly reproducible, with one exception, namely, interference with polymerase mRNA, in early preliminary experiments (data not shown). It has previously been reported that for lymphocytic choriomeningitis virus, transfection of siRNAs to L and Z mRNAs was ineffective, whereas targeting of the same gene products with siRNAs produced within the cells using an adenovirus expression system inhibited lymphocytic choriomeningitis replication efficiently (51). In our hands, the effect appeared to be especially sensitive to the transfection efficiency, depending on the experimental conditions and transfection reagents used (data not shown). Although L mRNA has the lowest concentration of viral mRNAs according to the transcriptional gradient and can be efficiently silenced, it is possible that in a fraction of incompletely transfected cells, a limited number of polymerases is sufficient to maintain viral replication. In experiments where nearly all cells were transfected and a homogenous distribution of the L siRNA within the cells was achieved, a strong and reproducible effect was exerted on viral gene expression. This was the case with the transfection reagent used to produce the data presented in this publication.

Interestingly, knockdown of the M protein not only enhanced the formation of syncytia, which was expected based on the findings previously reported for M-deficient recombinant MVs (13), but also increased the levels of other viral mRNAs and proteins and of the genomic RNA. The levels of both viral mRNAs and viral genomes were increased by a factor of 2 to 2.5 (Fig. 5). This suggests that the M protein hampers polymerase activity and that it affects mRNA and genome tran-

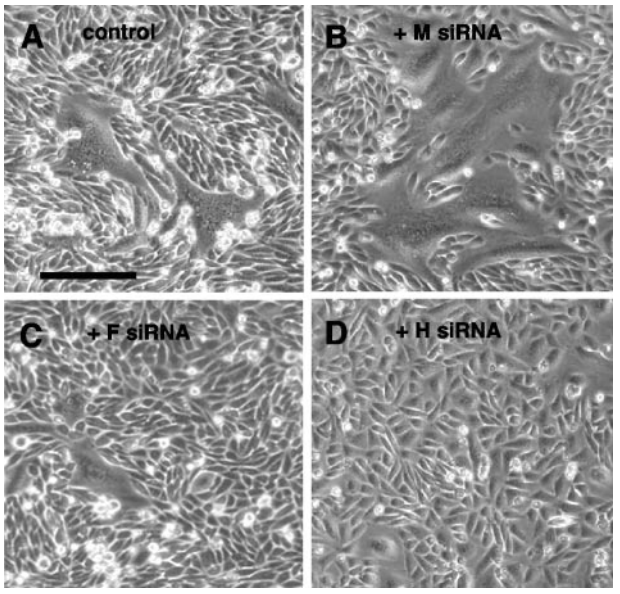

FIG. 3. Effect of envelope-specific siRNAs on syncytium formation. CHO-CD46 cells were transfected with control siRNA (A) and multiple siRNAs against M (B), F (C), and H (D) and infected with MV Edm at a multiplicity of infection of 0.5 for 48 h. Phase-contrast pictures reveal the number and extent of syncytia. (Magnification, ×20; bar in A, 20 μm.)

scription equally. Our findings are in concordance with previous findings showing that the M protein in in vitro transcription assays with purified RNP complexes causes a reduction of viral transcription (54). Binding affinities of M to the RNP did not appear to correlate with its inhibitory capacity, and those authors suggested that the functions of RNP binding and downregulation of transcription may be located in different domains of the M protein (54). In cells, the MV M protein expressed alone is distributed over the cell, whereas M in infected cells is localized at the plasma membrane and influences glycoprotein sorting and virus release (43, 49). Within the plasma membrane, lipid rafts appear to interact with the M protein in infected cells (40). Recent studies with MV and the closely related rinderpest virus demonstrated that the viral polymerase is also present at the plasma membrane (and in perinuclear complexes) (8, 17), where, as part of the RNP complex, it can interact with the M protein and where the inhibitory effect on viral transcription is probably exerted.

An inhibitory effect of M on transcription was observed with viruses such as Sendai virus, VSV, and influenza virus (12, 45, 58). In addition, as suggested by findings with VSV, the interaction of M with host cell factors may play a role (31, 34), and the M protein of human RSV is an RNA-binding protein (50). In the case of rabies virus, two different functions of M in the regulation of viral RNA synthesis, a pronounced inhibitory activity on viral mRNA transcription, and a simultaneous stimulatory activity on viral replication have been previously described (24). This is obviously not the case with measles virus, where we found that transcriptional and replicative activities of the polymerase are regulated in concordance.

Analyses of MV sequences in brains of patients suffering from subacute sclerosing panencephalitis have revealed that mutations accumulate predominantly in the envelope genes, most frequently in the M gene, which render the M mRNA nonfunctional (4, 5, 15). These mutations result in a defect of

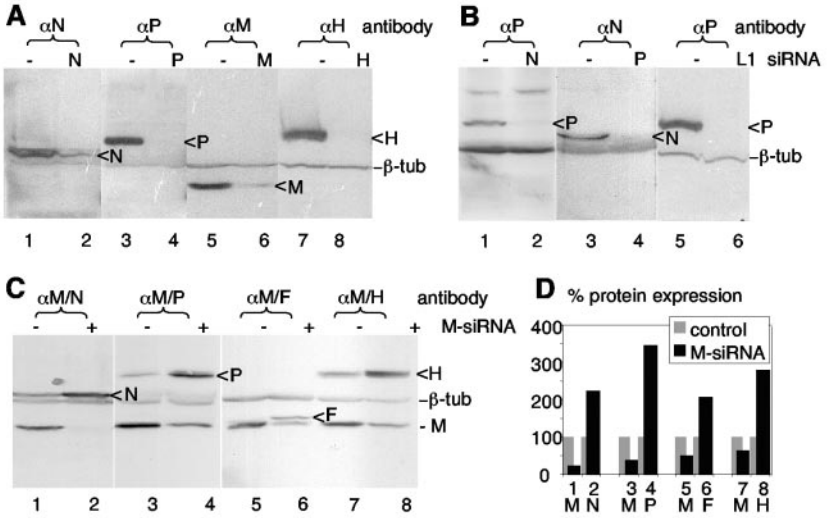

FIG. 4. *cis* and *trans* effects of siRNAs on the expression of viral proteins. CHO-CD46 cells were transfected with control siRNAs (−) or virus-specific siRNAs as indicated, infected, and lysed 48 h later, and proteins were separated by SDS-polyacrylamide gel electrophoresis and processed for Western blot analysis. Nitrocellulose filters were developed with antibodies against the proteins corresponding to the siRNAs (A), with antibodies against other proteins (B), or with a combination of M antibodies and antibodies against other viral proteins (C and D), as indicated on top of each panel. Arrowheads indicate viral proteins (N at 60 kDa, P at 70 kDa, M at 38 kDa, F at 40 kDa, and H at 78 kDa). β-Tubulin (β-tub) (at 50 kDa) was used as a control for the amount of protein blotted. In C, four independent experiments in which cells were infected with MV Edm and transfected with control siRNA (lanes 1, 3, 5, and 7) and M siRNA (lanes 2, 4, 6, and 8) are shown. In addition to antibodies against M, blots were stained with antibodies against N (lanes 1 and 2), P (lanes 3 and 4), F (lanes 5 and 6), H (lanes 7 and 8), and β-tubulin. The protein bands in C were quantified using a luminescent image analyzer, intensities of the bands of untreated cells were set to 100%, and band intensities from M siRNA-treated cells were set in relation to data in D.

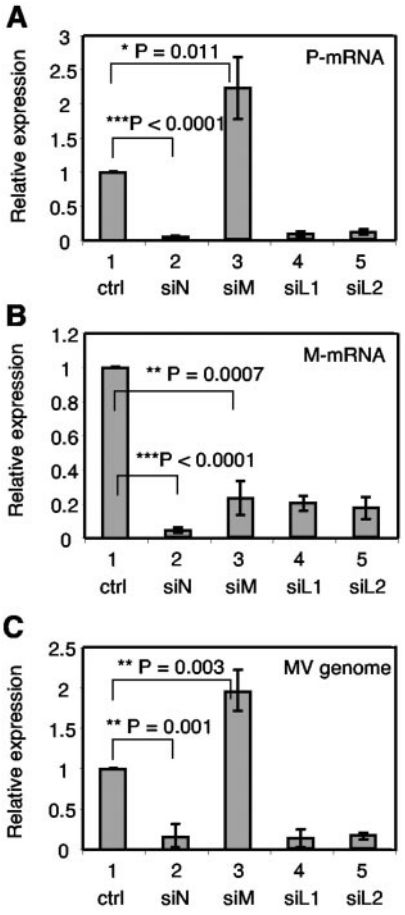

FIG. 5. Effect of siRNAs on viral mRNA and genomic RNA levels. RNAs from three independent experiments were analyzed by real-time RT-PCR, quantified as described in Materials and Methods, and averaged. (A) Relative expression levels of P mRNA in response to transfection of control siRNA (ctrl) (lane 1) and siRNAs against N (siN) (lane 2), M (lane 3), and L (lanes 4 and 5). (B) Relative expression levels of M mRNA in response to transfection of control siRNA (lane 1) and siRNAs against N (lane 2), M (lane 3), and L (lanes 4 and 5). (C) Relative expression levels of the viral genome in response to transfection of control siRNA (lane 1) and siRNAs against N (lane 2), M (lane 3), and L (lanes 4 and 5). All differences in the RNA levels between control and siRNA-treated cells are significant. Selected *P* values are shown.

viral assembly and budding. It has been speculated that mutations can occur due to the lack of a selective pressure, since only a small amount of or no functional envelope proteins seem to be required for the spread of viral RNPs from cell to cell in the brain. Receptor-independent mechanisms of virus spread, possibly as microfusion events at synapses, might circumvent the necessity for viral envelope proteins and cellular receptors (3, 18, 20, 37, 42, 55). In addition, it is likely that there is a positive selection for mutations in M, which may release the viral polymerase from control and support the accumulation and spread of infectious RNPs in the brain (4). Our findings of M as a transcriptional inhibitor support this hypothesis and indicate that not only the fusogenic activity of M-deficient viruses but also their replicative activity is enhanced. Previous attempts to cure a persistently MV-infected cell line (C6-SSPE) with antisense RNA expressing vectors expressing anti-N and anti-H RNA (35) have been successful in spite of the fact that the choice of these target sequences might have been suboptimal. For future therapeutic applications addressing this topic, RNAi might be a promising approach.

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